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Short communication

CCL28 involvement in mucosal tissues protection as a chemokine and as an antibacterial peptide

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ABSTRACT

CCL28 chemokine is expressed by epithelial cells of various mucosal tissues. This chemokine binds to CCR3 and CCR10 receptors and plays an essential role in the IgA antibody secreting cells (IgA-ASC) homing to mucosal surfaces and to lactating mammary gland as well. In addition, CCL28 has been shown to exert a potent antimicrobial activity against both Gram-negative and Gram-positive bacteria and fungi. Using the pig model, we investigated the expression of both CCR10 and CCR3 receptors in a large panel of mucosal tissues. RT-PCR analysis revealed the expression of CCR3 and CCR10 mRNA in salivary glands, nasal mucosae, Peyer's patches, small and large intestine, suggesting the presence of leucocytes expressing these receptors within these tissues. CCR10 mRNA was observed in sow mammary gland at late gestation with an increasing level during lactation. Recombinant porcine CCL28 protein was produced and mass spectrometry analysis revealed antimicrobial chemokines features such as a high pI value (10.2) and a C-terminal highly positively-charged region. Using a viable count assay, we showed that CCL28 displayed antimicrobial activity against enteric pathogens and was effective in killing *Salmonella* serotypes Dublin and Choleraesuis, enteroinvasive *Escherichia coli* K88 and non-pathogenic *E. Coli* K12. The potent antimicrobial function of CCL28 combined with its wide distribution in mucosal tissues and secretions suggest that this protein plays an important role in innate immune protection of the epithelial surfaces.

1. Introduction

Chemokines constitute a superfamily of low-molecular-weight chemotactic cytokines (8–13 kDa) primarily recognized for their role in lymphocyte trafficking *via* seven transmembrane-spanning G protein-coupled receptors (GPCR) located on the cell surface (Williams, 2004). Additionally, recent evidence suggests a critical role for chemokines in a variety of pathophysiological processes such as infectious and autoimmune diseases, allergic responses, chronic and acute inflammation (Horuk, 2007; Raman et al., 2011). In addition to their function in cell homing and migration, several chemokines have been shown to exhibit direct antimicrobial activity against Gram-negative and Gram-positive bacteria (Yang et al., 2003). However, our understanding of their antimicrobial defence involvement is still rudimentary as compared with their primary role in leucocytes trafficking. In a previous study, the assessment of 11 human recombinant chemokines panel demonstrated that the three interferon-γ (INF-γ)-inducible chemokines, including monokine induced by IFN-γ (MIG/CXCL9), IFN-γ inducible protein of 10 kDa (IP-10/CXCL10) and IFN-inducible T-cell α chemoattractant (I-TAC/CXCL11) were antimicrobial against *Escherichia coli* and *Listeria Monocytogenes* (Cole et al., 2001). Furthermore, the antimicrobial activity of 30 human chemokines panel was tested *in vitro* against *E. Coli*, and *Staphylococcus aureus* and 18 chemokines were identified as exhibiting the capacity to kill the bacteria (Yang et al., 2003). Several antimicrobial chemokines have been shown to be expressed at the epithelial surface, representing an important component of the mucosal innate immunity. Recently, CXCL14 chemokine was shown to be highly expressed in the epidermis and dermis of human skin and to exhibit killing activity against *E. Coli* and cutaneous Gram-positive bacteria such as coagulase-negative *Staphylococcus* spp., *Staphylococcus aureus* and *Propionibacterium* spp. (Maerki et al., 2009). In addition, CXCL9, CXCL10 and CXCL11 are produced by inflamed pharyngeal epithelial cells and mediate *Streptococcus pyogenes* killing suggesting a role in streptococcal pharyngitis protection (Egesten et al., 2007). Both CXCL6 and CXCL9 are constitutively expressed by epithelial cells of the male urogenital tract and possess antimicrobial activity against urogenital pathogen *Neisseria gonor-*

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rhoeae (Linge et al., 2008; Collin et al., 2008). Similarly, CCL20 is secreted by the female reproductive tract epithelium at levels that were found to be inhibitory of HIV-1 virus (Ghosh et al., 2009). Altogether, these data suggest that antimicrobial chemokines function both to recruit leucocytes to specific mucosal tissues as well as to protect these surfaces through direct antimicrobial activity.

Mucosa-associated Epithelial Chemokine (MEC)/CCL28 is produced constitutively by epithelial cells within a variety of human and mice mucosal tissues including small and large intestine, the bronchial, salivary and mammary glands (MG) (Pan et al., 2000; Wang et al., 2000). This chemokine mediates the trafficking and the accumulation of the immune cells expressing CCR3 and/or CCR10 receptors in intestinal and extra intestinal sites (Pan et al., 2000; Wang et al., 2000; Kunkel et al., 2003; Hieshima et al., 2003). CCR10 is expressed on IgA antibody secreting cells (IgA-ASC) and CCL28/CCR10 interaction was identified as a key regulator in the recruitment and the homeostatic localization of IgA-ASC within mouse lactating MG (Wilson and Butcher, 2004; Morteau et al., 2008). CCL28 protein was found in both human milk and saliva (Hieshima et al., 2003), and exerts a potent antimicrobial activity against a broad range of microbes including *Pseudomonas aeruginosa, Streptococcus mutans*, *Klebsiella pneumoniae, Porphyromonas gingivalis, Actinobacillus actinomycetemcomitans* and *Candida albicans* (Hieshima et al., 2003; Watkins et al., 2007; Liu and Wilson, 2010). Although the antimicrobial activity of several chemokines has been described, the precise mechanism by which the microbicidal function is exerted is still unknown. A mechanism for the antimicrobial activity of defensins and other antimicrobial peptides has been proposed in which the C-terminal cationic region binds, via electrostatic interactions, to the negatively-charged membrane surface, ultimately resulting in pore formation and leaks of cellular contents of the target microbes (Hieshima et al., 2003; Liu and Wilson, 2010). To the extent that epithelial cell production of CCL28 influences mucosal IgA B cell migration and, similar to other chemokine, mediates antimicrobial activity, CCL28 could contribute to regulation of host mucosal defence at homeostasis as well as under infection or inflammation. However, there is a paucity of information regarding these dual functions of CCL28 in mucosal immunity of large animals (Meurens et al., 2006; Berri et al., 2008; Distelhorst et al., 2011). Using the pig model, we previously cloned CCL28 chemokine and showed its expression in a variety of porcine epithelial tissues, including small and large intestine, trachea, tonsil, nasal mucosae, salivary and lactating MG (Meurens et al., 2006; Berri et al., 2008). In the present study, we set out a comparative analysis of mRNA expression of CCL28 receptors, CCR10 and CCR3 in a large panel of 14 mucosal tissues and assessed also their expression kinetic during MG development. Moreover, because the involvement of antimicrobial chemokines in the gastrointestinal tract is still unknown and CCL28 antimicrobial activity against intestinal pathogens has never been tested, we investigated the possibility that this chemokine would have defensive activity against enteric pathogens such as different *Salmonella* serotypes, and enteroinvasive *E. Coli* which are bacteria with a high economic impact on swine production systems and a threat to safety of pork products as well. Our results regarding dual CCL28 involvements in both directing IgA-ASC and as antimicrobial peptide against enteric pathogens will help in designing new relevant therapeutic interventions.

2. Materials and methods

2.1. Animals and tissue samples

Three old-months healthy histocompatible miniature pigs (SLA d/d) (Kaeffer et al., 1991) were used. The Animal care and conduct of the study were performed under the approval and guidelines of the Institutional Animal Care and Use committee at INRA (France). Pigs were electrocuted and exsanguinated in a local slaughterhouse. A set of two samples (100–150 mg) of chosen were taken immediately after animal slaughter, snap-frozen in liquid nitrogen and stored in −70 °C until used. In addition, biopsies of sow mammary gland (MG) were taken at 50, 83, 113 days of gestation and at 3, 9, 16, 20 days of lactation. The animals were anesthetized with 5 ml of stressnil (Janssen, Issy Les Moulineaux, France) and a local anesthesia procedure was added using 2 ml of Lurocaïne, injected subcutaneously (Vetoquinol, Lure, France). The biopsies were obtained using a 4 mm biopsy punch instrument (V Kruuse Company, Marslev, Denmark), mixed with 1 ml of Trizol reagent (Invitrogen, Cergy, France) and used immediately for RNA preparation.

2.2. RT-PCR analysis of CCR10 and CCR3 receptors in pig tissues

The cDNA of porcine CCR10 receptor was previously cloned (Meurens et al., 2006) and found to be 1092 nucleotides in length, encoding a predicted precursor protein of 363 amino acids (GenBank Accession number BC099840). Porcine CCR3 receptor cDNA was already available in the GenBank data base under the Accession number of NM_001001620. A total RNA was prepared by homogenization of 0.1–0.2 g pig tissue in 1 ml of Trizol reagent (Invitrogen, Cergy, France) according to the manufacturer's recommendations, then semi-quantitative RT-PCR analysis was performed as previously described (Bourges et al., 2004). A pair of primers was designated based on CCR10 (NCBI Accession No. BC099840), CCR3 (NCBI Accession No. NM_001001620) and cyclophilin (NCBI Accession No. NM_001243664) sequences. This former was used as constitutively expressed gene for RNA content and integrity and to check the uniformity of the RT reactions. CCR10 forward: 5′ CTGTTGGAATACTGTCGACCTAC 3′; CCR10 reverse: 5′ AGAAGGAGACACTGTTGGTCT 3′. CCR3 forward: 5′ GGAATCATTAAAAACACTGCTG 3′ CCR3 reverse 5′ CTTTCACTAG GAATAAATGGGATGA3′. Cyclophilin forward: 5′ TAACCC-CACCGTCTTCTT 3′; Cyclophilin reverse: 5′ TGCCATCCAACCACTCAG 3′. Amplification products (10 μl of each) were separated by electrophoresis in a 2% TBE agarose gel stained with ethidium bromide and visualized on a UV transilluminator. We determined the fluorescence intensity of bands with an Alpha Fluorchem Imager Gel Analysis System version 2.00 (Alpha Innotech Corporation, San Leandro, CA, USA). All samples were tested in triplicate and the bands intensity of amplified chemokines was normalized with those of corresponding cyclophilin.

2.3. Expression and purification of recombinant CCL28 protein

Complementary DNA of CCL28 ORF lacking the peptide signal sequence was previously cloned in pGMET vector and consists of 315-bp encoding 105 amino acids protein. This region was amplified from pGMET-CCL28 recombinant plasmid, cloned into the expression plasmid pQE-30 (Qiagen, S.A., Courtaboeuf, France) and produced as an N-terminally six-histidine-tagged fusion protein using the QIAexpressionist kit (Qiagen, Courtaboeuf, France) (Berri et al.; 2008). Briefly, the recombinant plasmid pQE30-CCL28 was transformed into *E. Coli* TG1 strain and recombinant 6 × His-CCL28 was expressed after induction by 1 mM of isopropyl-β-D-1-thiogalactopyranoside (IPTG) for 4 h. The bacteria were lysed with lysozyme (1 mg/ml), sonicated in lysis buffer (20 mM Tris–HCl, pH 8.0, 100 mM KCl, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride, and protease inhibitors), and the supernatant-containing CCL28 fusion protein was subjected to nickel-nitrilotriacetic acid affinity gel column chromatography (Qiagen, Courtaboeuf, France). The protein concentration was determined using BCA assay kit (Thermo Scientific Pierce, Rockford, IL, USA) and